Engineered viral vaccine constructs with dual specificity: Avian influenza and Newcastle disease

Man-Seong Park*, John Steel*, Adolfo García-Sastre*, David Swayne†, and Peter Palese*‡

*Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029; and †Southeast Poultry Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Athens, GA 30605-2195

Contributed by Peter Palese, March 29, 2006

Avian influenza viruses of the H5 and H7 hemagglutinin subtypes, and Newcastle disease virus (NDV), are important pathogens in poultry worldwide. Specifically, the highly pathogenic H5N1 avian influenza virus is a particular threat because it has now occurred in more than 40 countries on several continents. Inasmuch as most chickens worldwide are vaccinated with a live NDV vaccine, we embarked on the development of vaccine prototypes that would have dual specificity and would allow a single immunization against both avian influenza and Newcastle disease. Using reverse genetics, we constructed a chimeric avian influenza virus that expressed the ectodomain of the hemagglutinin-neuraminidase gene of NDV instead of the neuraminidase protein of the H5N1 avian influenza virus. Our second approach to creating a bivalent vaccine was based on expressing the ectodomain of an H7 avian influenza virus hemagglutinin in a fusogenic and attenuated NDV background. The insertion into the NDV genome of the foreign gene (containing only its ectodomain, with the transmembrane and cytoplasmic domains derived from the F protein of NDV) resulted in a chimeric virus with enhanced incorporation of the foreign protein into virus particles. A single immunization of chickens with this improved vaccine prototype virus induced not only a 90% protection against an H7N7 highly pathogenic avian influenza virus, but also complete immunity against a highly virulent NDV. We propose that chimeric constructs should be developed for convenient, affordable, and effective vaccination against avian influenza and Newcastle disease in chickens and other poultry.

chicken | Newcastle disease virus | pandemic | avian influenza virus | hemagglutinin

In recent years, outbreaks of high-pathogenicity avian influenza (HPAI) have been reported in Asia and Europe (1, 2). These outbreaks involving H5N1 or H7N7 influenza viruses resulted in lethal infections in domestic poultry and the death of a limited number of people (2–4). The current H5N1 viruses have been circulating among poultry in China in recent years (5, 6) and, although migratory birds are considered to be the primary reservoir of these viruses, transmission from infected poultry back to migratory birds is believed to have contributed to the increased geographical distribution of the viruses (7). Currently, the H5N1 virus has emerged from Asia, spreading across Europe and Africa (8). Wholesale culling of poultry was shown to be a successful strategy for eradicating H5N1 outbreaks in Hong Kong in 1997 and in The Netherlands in 2003 (9). Because human victims of recent HPAI outbreaks have had close contact with infected poultry (10), it follows that the prevention of interspecies transmission of avian influenza viruses (AIVs) may be accomplished by eradicating AIV in poultry through slaughter. However, for economic and practical reasons, the destruction of infected poultry alone is no longer considered the method of choice for the control of this disease. In addition, for ethical and ecological reasons, the culling of migratory wildfowl is considered an unacceptable practice. Recently, the World Organization for Animal Health and the Food and Agriculture Organization of the United Nations recommended that vaccination of poultry be considered for the control of AIV (11). In addition, it has been reported that vaccination of chickens with inactivated H5 vaccine in a field study was successful in interrupting virus transmission (12). Recently, China has accepted vaccination as a component of its AIV control program. Accordingly, the entire poultry population in China is to be vaccinated (13).

As part of our approach to the development of vaccines with dual specificity against AIV and Newcastle disease virus (NDV), we report here on the construction of an avian H5 influenza virus in which the ectodomain of the avian neuraminidase (NA) is replaced with that of the hemagglutinin-neuraminidase (HN) protein of an NDV. The latter protein is the major antigen of NDV that has been shown to elicit a protective immune response in chickens (14, 15). Construction of this virus was made possible by using the reverse genetics technology described in ref. 16. A bivalent influenza virus expressing the parainfluenza HN ectodomain was also reported recently (17).

Our second approach to the development of a bivalent vaccine is based on the NDV platform. NDV is a highly contagious avian disease agent of the genus *Avulavirus*, belonging to the family *Paramyxoviridae* and can vary in virulence (18). Over 250 species of birds, including poultry, are susceptible to NDV (19). Mortality from either highly virulent NDV or HPAI infection can reach up to 100% in unvaccinated poultry flocks. Therefore, the development of a combined vaccine against AIV and NDV would be of benefit to the poultry industry and would improve public health by reducing the HPAI load in vaccinated poultry below the level of transmissibility to people.

We and others have previously established reverse genetics systems to manipulate the genome of NDV (20, 21, 31, 32), allowing the generation of recombinant NDVs (rNDVs) that express foreign proteins. We have previously shown that immunization with rNDV expressing the influenza A/WSN/33 (H1N1) virus (WSN) hemagglutinin (HA) protein protected mice from challenge with a lethal dose of WSN (21). On the basis of these results, we first attempted to develop a bivalent vaccine against NDV and HPAI H7 virus by using a nonfusogenic rNDV vector expressing the WT HPAI H7 HA protein (rNDV/B1-H7). However, immunization of chickens with this rNDV/B1-H7 induced only partial immunity against HPAI and highly virulent NDV (22). Here, we have developed and evaluated a bivalent vaccine candidate against both AIV and NDV for poultry that is based on the fusogenic rNDV vector expressing a chimeric HA protein. This vaccine shows improved protection against both pathogens in a chicken challenge model.

Conflict of interest statement: Mount Sinai School of Medicine owns patent positions for reverse genetics of negative-strand RNA viruses (A.G.-S. and P.P.).

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Abbreviations: HPAI, high-pathogenicity avian influenza; AIV, avian influenza virus; NDV, Newcastle disease virus; NA, neuraminidase; HN, hemagglutinin–neuraminidase; rNDV, recombinant NDV; HA, hemagglutinin; WSN, influenza A/WSN/33 (H1N1) virus; cHN, chimeric HN; EID_{50}, egg 50% infectious dose; MDCK, Madin–Darby canine kidney; vRNA, viral RNA; CEF, chicken embryo fibroblast; MDT, mean death time; F, fusion protein; HI, hemagglutination inhibition; pNDV, parental NDV; vNDV, velogenic NDV.

‡To whom correspondence should be addressed. E-mail: peter.palese@mssm.edu.

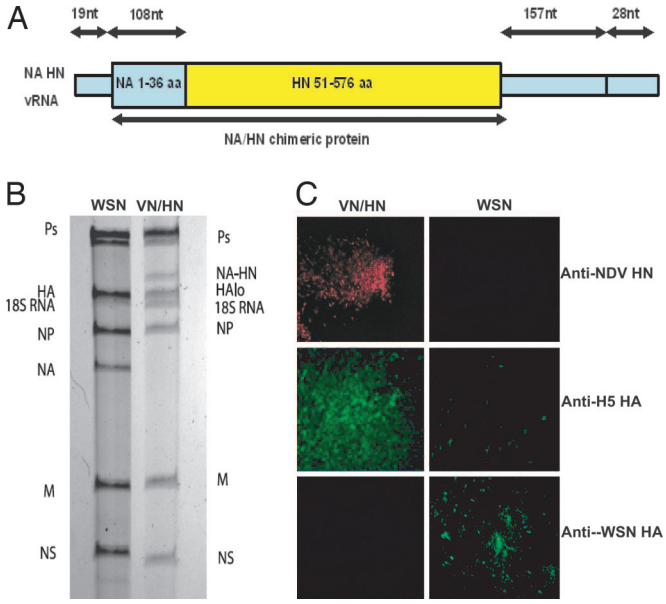

Fig. 1. Generation of a transfectant influenza virus bearing a chimeric NA-HN segment. (*A*) Schematic diagram of the viral RNA (vRNA) encoding the influenza virus NA NDV HN chimeric segment. The vRNA consists of the first 127 nucleotides of the WSN NA vRNA, which includes the 19 nucleotides of the noncoding region and the nucleotides encoding the cytoplasmic tail, the transmembrane domain, and the start of the NA stalk. This domain is followed by the nucleotides encoding amino acids 51–576 of the NDV HN protein, comprising the HN ectodomain and tandem stop codons (TAG TAG). The last domain represents the 185 nucleotides of the 5′ end of the ORF of the WSN NA (157 nucleotides) and of its 5′ noncoding region (28 nucleotides). (*B*) vRNAs of purified recombinant VN/HN virus. RNAs extracted from recombinant WT WSN and VN/HN viruses were separated through a 2.8% polyacrylamide gel containing 7 M urea and were visualized by silver staining. The positions of the polymerase (Ps), HA (WSN), HAlo (VN 1203), NP, M, and NS segments are indicated. The RNA encoding the NA protein is absent from the VN/HN virus. A contaminating 18S ribosomal RNA band is indicated. (*C*) Expression of cHN protein. MDCK cells were infected with influenza VN/HN virus or with WT WSN. Infected cells were incubated for 16 hr at 37°C, fixed, and permeabilized with methanol. Specific antigens present in the virus were detected by immunofluorescence with anti-NDV HN monoclonal antibody (7B1), anti-influenza H1 HA monoclonal antibody (2G9), and polyclonal anti-influenza H5 HA serum.

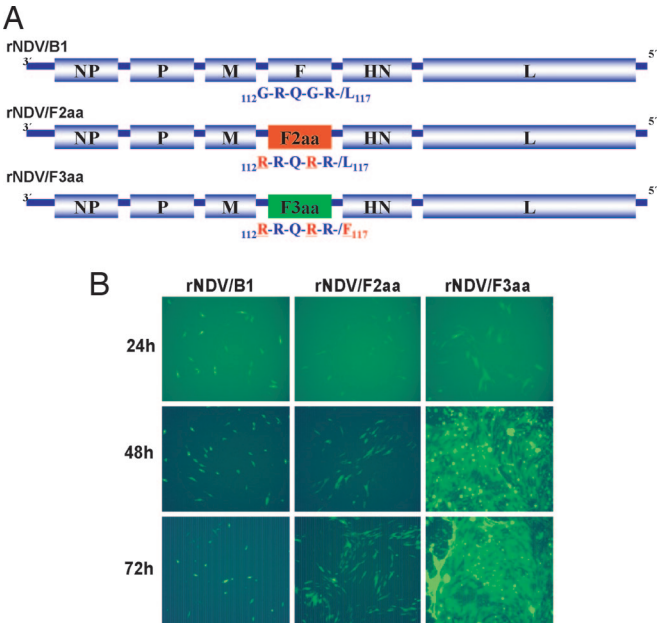

Fig. 2. Modification of the cleavage site of the F protein of rNDV. (*A*) Schematic representation of the rNDV/B1 genome with two or three amino acid changes in the cleavage site of their F proteins. The modified cleavage sites are known to be recognized by ubiquitous proteases. The peptide bond that is cleaved in the F protein is indicated with a slash. (*B*) Syncytia formation in CEF cells infected by rNDVs with modified F proteins. CEF cells were infected, with a multiplicity of infection of 0.001, with rNDV/B1, rNDV/F2aa, and rNDV/F3aa viruses. Infected cells were incubated at 37°C, and then viral spreading by cell fusion was monitored every 24 hr by immunofluorescence assay.

Results

Generation of a Virus Expressing Influenza Virus and NDV Antigens. To obtain a candidate live bivalent vaccine virus for protection against both avian H5 influenza and NDV, we constructed a transfectant influenza virus (designated VN/HN virus) with a chimeric segment in which the encoded NA ectodomain, responsible for NA activity, was replaced with the ectodomain from the NDV HN protein (cHN) (Fig. 1*A*). Because of their importance in the formation of infectious virus (23, 24), the cytoplasmic tail and transmembrane domains of the influenza NA protein were retained. Numerous reports have indicated that the polybasic cleavage peptides of the H5 HA and H7 HA proteins are required for high pathogenicity of viruses in chickens (25). Thus, as a strategy to attenuate the transfectant virus while retaining the H5 antigen, we removed the polybasic cleavage site and replaced it with a consensus sequence derived from avirulent H5 HA strains. The introduction of adenosine residues into the nucleotide sequence encoding the HA cleavage peptide of avirulent H5 influenza strains has been associated with spontaneous conversion to virulence. This introduction is postulated to occur through a mechanism of polymerase slippage (26); therefore, to minimize the opportunity for the reintroduction of adenosine residues, we altered the codon usage in the region of the cleavage peptide, resulting in the formation of an avirulent HA consensus sequence designated HAlo. A transfectant virus containing HAlo and cHN expressing segments derived from constructs encoding these alterations was generated by a modified version of the cDNA-based rescue of Fodor *et al.* (16).

To be of use as a vaccine, a virus must grow efficiently in eggs or cell culture. The recombinant virus was amplified in 10-day-old embryonated chicken eggs and grew to a titer of 8.0 $\log_{10}$ egg 50% infectious dose (EID_{50}) per milliliter in this substrate. In Madin–Darby canine kidney (MDCK) cell culture, the virus grew to a titer of 5×10^8 plaque-forming units per milliliter and produced plaques approximately two-thirds of the size of WSN.

The incorporation of viral RNA (vRNA) into the chimeric viral particle was examined by acrylamide gel electrophoresis of purified virion RNA. The presence of eight viral segments was observed, with the chimeric HN segment, which has a predicted size of 1,911 nt, migrating between the PA and HAlo segments (Fig. 1*B*). The identity of the HA and cHN segments was confirmed by RT-PCR, and the segments were sequenced by using segment-specific primers. Incorporation of the cHN segment was shown to be stable for eight passages in embryonated chicken eggs by acrylamide gel electrophoresis of purified virion RNA (data not shown). Furthermore, expression of the recombinant HAlo and cHN proteins during viral infection was confirmed by immunofluorescence. MDCK cells infected with VN/HN virus expressed HAlo and cHN proteins 16 hr after infection, whereas, in cells infected with the control WSN, HA but not HN protein was expressed (Fig. 1*C*).

Generation of Fusogenic rNDV Mutants. Previously, a nonfusogenic, rNDV virus (rNDV/B1) expressing the H7 HA protein was unable to generate the required immunogenicity to completely protect chickens against H7 HPAI and highly virulent NDV (22). To overcome this limitation, we have developed two rNDV mutants, rNDV/F2aa and rNDV/F3aa, in which the cleavage site of the F protein was replaced with one containing one or two extra arginine

Table 1. MDT of rNDVs in embryonated chicken eggs

Virus	Trypsin requirement (cell culture)	Inoculation, EID_{50}	MDT, hr
rNDV/B1	Yes	10	113
		1	122
rNDV/F2aa	No	10	100
		1	104
rNDV/F3aa	No	10	80
		1	84
rNDV/B1-H7	Yes	10	Alive
		1	Alive
rNDV/F3aa-chimericH7	No	10	128
		1	140

MDT has been used to classify NDV strains. Velogenic NDV strains take <60 hr to kill the embryos, whereas mesogenic NDV strains and lentogenic NDV strains take 60–90 hr and >90 hr, respectively.

residues (Fig. 2*A*). These cleavage sites can be activated by ubiquitously expressed proteases of the furin family (27). Infection of chicken embryo fibroblast (CEF) cells with rNDV/F2aa and rNDV/F3aa, and not with rNDV/B1, resulted in the formation of syncytia (giant multinucleated cells that aid in the spread of the virus) in the absence of exogenously added protease (Fig. 2*B*). In addition, rNDV/F3aa induced syncytia more rapidly in CEF cells than did rNDV/F2aa. Thus, it was postulated that improved spreading of the virus in immunized animals may enhance immunogenicity against the inserted foreign protein. Hence, we selected the fusogenic rNDV/F3aa as a backbone vector to develop a bivalent vaccine designed to protect poultry against AIV and NDV.

Mean Death Time (MDT) Analysis of rNDV Platform Vectors in Embryonated Chicken Eggs. NDV can be classified as highly virulent (velogenic), intermediate (mesogenic), or low-virulent (lentogenic) on the basis of its pathogenicity for chickens. Because the presence of a fusion protein (F protein) with a multibasic cleavage site is known to be an NDV virulence factor, we assessed the pathogenicity of rNDVs with modified F protein in 10-day-old embryonated chicken eggs. The MDT of chicken embryos infected with NDVs correlates with virulence *in vivo* (19). Lentogenic strains, which cause asymptomatic infections in birds, are characterized by MDTs of >90 hr; mesogenic strains, which cause respiratory disease in birds, have MDTs between 60 and 90 hr; and velogenic strains, which cause severe disease in birds, have MDTs of <60 hr. The MDT of rNDV/F2aa was indicative of a lentogenic strain, whereas that of rNDV/F3aa was typical of a mesogenic strain. Neither of these strains had MDTs typical of a highly virulent (velogenic) strain (Table 1). On the basis of these data, rNDV/F3aa vector would represent a lower threat to birds and is thus suitable as a backbone to develop a bivalent vaccine for the protection of poultry against AIV and NDV.

Generation of a Fusogenic rNDV Vector Expressing the Ectodomain of AIV HA Protein. To construct a chimeric HA gene for insertion into a fusogenic rNDV, the ectodomain of the H7 HA protein from A/chicken/NY/13142-5/94 (H7N2) was fused with the transmembrane domain and cytoplasmic tail regions derived from the NDV F protein. This chimeric H7 HA gene was then inserted between the P and M genes of the rNDV/F3aa vector, resulting in the formation of rNDV/F3aa-chimericH7 (Fig. 3*A*). rNDV/F3aa-chimericH7 virus was rescued from cDNA by using the methods described in ref. 21. The presence of the inserted chimeric H7 HA gene in the viral genome was confirmed by RT-PCR and sequencing (data not shown). The growth kinetic of rNDV/F3aa-chimericH7 in embryonated chicken eggs was compared with that of the parental rNDV/F3aa (Fig. 3*B*). The virus expressing the chimeric H7 HA protein grew more slowly than the virus without the insert, and

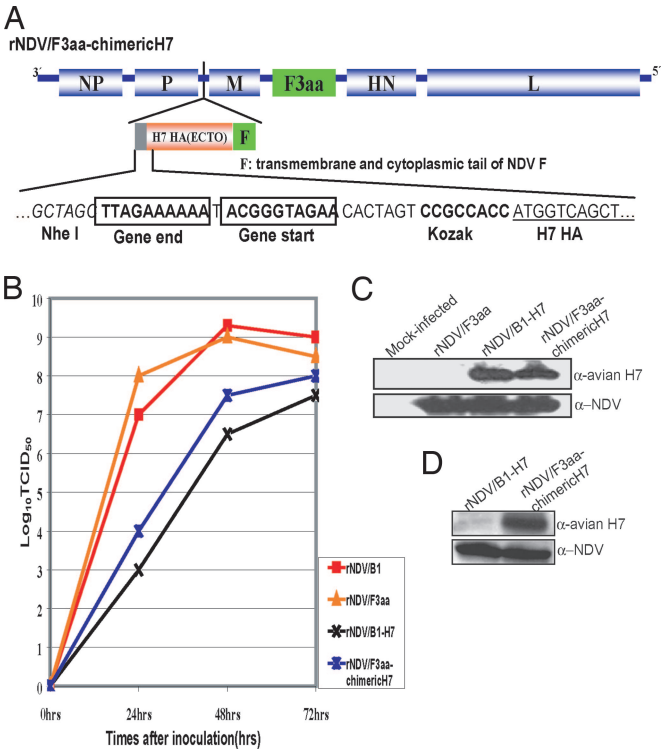

Fig. 3. Construction and characterization of the fusogenic rNDV vector expressing HPAI H7 HA protein. (*A*) Schematic representation of rNDV/F3aa-chimericH7 cDNA construct. The ectodomain of the H7 HA protein was fused with the transmembrane domain and cytoplasmic tail of the NDV F protein. This chimeric H7 HA gene was inserted between the P and M genes of the rNDV/F3aa vector. (*B*) Comparison of viral growth kinetics. Ten-day-old embryonated chicken eggs were inoculated with 100 plaque-forming units of each virus, and allantoic fluids were harvested at different time points (24, 48, and 72 hr after inoculation). Viral titers (tissue culture 50% infective dose, $TCID_{50}$) in Vero cells were determined by immunofluorescence assay with an anti-NDV rabbit serum and an anti-rabbit IgG FITC-labeled antibody (DAKO). (*C*) Expression of the WT H7 HA protein or the chimeric H7 HA protein in cells infected with rNDVs. Infected cells were harvested at 36 hr after infection, and cell lysates were used for immunoblotting analysis. (*D*) Incorporation of chimeric H7 HA protein into rNDV virions was increased as compared with that of WT H7 HA protein. rNDV/B1-H7 and rNDV/F3aa-chimericH7 were purified from allantoic fluids of infected embryonated chicken eggs. The viral proteins from rNDV/B1-H7 or rNDV/F3aa-chimericH7 were separated on an SDS/10% polyacrylamide gel. The viral proteins were transferred to a nitrocellulose membrane, and the H7 HA proteins were detected by chemiluminescence with an anti-chicken H7 HA polyclonal antibody and an anti-chicken IgG peroxidase-labeled antibody (Jackson ImmunoResearch).

maximal titers were about a log lower. Interestingly, the MDT of this virus was that of a lentogenic strain (≈128–140 hr) (Table 1). Expression of the chimeric H7 HA protein from rNDV/F3aa-chimericH7 was confirmed by Western blotting of infected Vero cells 36 hr after infection (Fig. 3*C*).

Improved Incorporation of AIV H7 HA Protein into rNDV Virions. We postulated that expression of the chimeric H7 HA protein containing the heterologous transmembrane and cytoplasmic tail regions of the NDV F protein would be associated with enhanced incorporation into rNDV virions. To address this question, rNDV/B1-H7 and rNDV/F3aa-chimericH7 virions were purified as described in *Materials and Methods*. The amounts of H7 HA protein or NDV viral protein from rNDV/B1-H7 or rNDV/F3aa-chimericH7 were measured by Western blotting using anti-chicken AIV H7 polyclonal antibody or anti-rabbit NDV polyclonal serum. As expected, incorporation of chimeric H7 HA protein into rNDV virions was significantly increased compared with that of WT H7

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Table 2. HI serology and survival of chickens before and after challenge

Vaccine group*	HI serology before challenge (GMT)		Challenge virus	No. of survivors	HI serology 14 days after challenge (GMT)	
	AIV/H7 antigen	NDV antigen			AIV/H7 antigen	NDV antigen
rNDV/F3aa-chimericH7, 1×	8/10 (11)	10/10 (49)	vNDV	10/10	9/10 (15)	10/10 (416)
rNDV/F3aa-chimericH7, 1×	7/10 (10)	10/10 (49)	HPAIV	9/10	9/9 (2,048)	9/9 (37)
rNDV/F3aa-chimericH7, 2×	8/10 (13)	9/10 (56)	vNDV	10/10	7/10 (17)	10/10 (315)
rNDV/F3aa-chimericH7, 2×	5/10 (9)	9/10 (60)	HPAIV	9/10	8/8 (955)	8/8 (30)
pNDV, 2×	0/10	10/10 (34)	vNDV	10/10	0/10	10/10 (294)
pNDV, 2×	0/10	10/10 (56)	HPAIV	0/10	NA	NA
Sham, 2×	0/10	0/10	vNDV	0/10	NA	NA
Sham, 2×	0/10	0/10	HPAIV	3/10	0/3	0/3

Chickens were vaccinated with rNDV containing the AIV H7 HA gene (rNDV/F3aa-chimericH7), pNDV, or sterile tissue culture fluid (sham), followed by challenge with vNDV and A/Human/Steele/59 (H7N7) HPAI virus (HPAIV). HI serology is shown as no. of chickens with HI-positive serum/no. of chickens vaccinated; values in parentheses are geometric mean titer (GMT). NA, not applicable.
*n = 10 birds per group; 1× = one vaccination; 2× = two vaccinations.

HA protein (Fig. 3*D*). These data suggest that the transmembrane and cytoplasmic tail regions of the NDV F protein play a major role in the improved incorporation of the foreign protein into the viral surface.

Immunization and Challenge of Chickens. After one or two vaccinations with rNDV/F3aa-chimericH7, 50–80% of the chickens inoculated had hemagglutination inhibition (HI) titers to H7 AIV, and 90–100% of the chickens had HI titers to NDV (Table 2). In contrast, all chickens immunized twice with the parental NDV/B1 (pNDV) had HI titers to NDV but none had titers to H7 AIV. All sterile tissue culture media (sham)-infected birds lacked HI titers to either virus. When challenged with a highly virulent strain of NDV (velogenic NDV, vNDV), 100% of rNDV/F3aa-chimericH7- and pNDV-immunized chickens were protected. By comparison, 90% of rNDV/F3aa-chimericH7-vaccinated chickens were protected from HPAI H7 virus, but none of pNDV-vaccinated chickens were protected from HPAI H7 virus. By contrast, 100% and 70% of sham-infected birds died when challenged by vNDV and HPAI H7 virus, respectively. The survivors mounted an anamnestic response evidenced as a 4-fold or greater rise in HI titer for the respective challenge virus, except for the three survivors in the sham-HPAI H7-virus challenge group which had no serological evidence of being infected.

Discussion

Vaccination against AIV in poultry can play an important role in reducing virus shedding and raising the threshold for infection and transmission. It is believed that vaccination with a high-quality vaccine against AIV can be part of an effective control program. In conjunction with culling, quarantine, improved serological surveillance, and high biosecurity, the control of outbreaks of HPAI in poultry, as well as the prevention of transmission of HPAI to humans, is possible. Because the vaccination of poultry with live vaccines against NDV is mandatory in many countries (19), the development of bivalent vaccines should be made a priority. Such vaccines would reduce the burden of vaccine production and administration in comparison with individual vaccination. For these reasons, we have focused on the development of live bivalent viruses that show potential as vaccines against the major economic pathogens AIV and NDV.

First, by reverse genetics, we produced an influenza virus (VN/HN) that expresses the ectodomain of the NDV HN protein in place of the influenza virus NA. The HN of NDV has been shown to be the major antigen eliciting a protective immune response in chickens (14, 15). We reasoned that the HN protein would confer protection against NDV and provide the necessary NA activity in the absence of the endogenous NA protein. The VN/HN virus grew efficiently in embryonated chicken eggs and stably expressed the cHN segment, both of which characteristics are fundamental requirements for a successful vaccine. However, preliminary experiments using VN/HN to vaccinate 2-week-old White Leghorn chickens revealed that this virus is possibly too attenuated *in vivo* to induce a highly protective immune response in chickens (data not shown). Experiments are needed to examine the ability of VN/HN to induce a protective response after immunization of chick embryos *in ovo*. Such an approach requires a more highly attenuated vaccine strain than that used for vaccination of hatched chickens.

Next, we chose NDV as a bivalent vaccine vector because it possesses several properties that make it suitable for use in viral vaccine development. The RNA genome of the virus does not integrate into host cell DNA. Furthermore, the rNDV vector can stably incorporate an inserted foreign gene over multiple passages and, because it is a respiratory virus, it can provide a convenient platform for rapid, efficient, and economical mass immunization of poultry. These benefits are multiplied in the case of a multivalent vaccine; one immunization can lead to protection from two or more pathogens. Whereas simultaneous vaccination with multiple live attenuated viruses may lead to complications if the presence of one virus interferes with the growth or immunogenicity of another, such problems are circumvented entirely by the use of a single bivalent virus vaccine. Thus we propose that, by using recently developed reverse genetics techniques (20, 21), it is possible to develop a new generation of effective, economical, and convenient bivalent live attenuated vaccines.

An outbreak of HPAI H7N7 in The Netherlands in 2003, in which 30 million chickens were slaughtered, involved the transmission of H7N7 to 89 persons and led to the death of a veterinarian. In addition, an HPAI H7N3 virus emerged in Canada in 2004 that also transmitted to humans (2, 3). Accordingly, in the present study we produced rNDV that expressed the H7 HA protein, with the intention of protecting poultry from HPAI H7 and NDV, and showed its efficacy in chickens. We suggest that the HAs from genetic variants of HPAI H5N1 or HPAI H7N7 viruses, as well as from other potential pandemic strains such as H9N2, could be inserted into the rNDV fusogenic vector to develop bivalent vaccines against these AIV strains and NDV. In addition, the rNDV vector can potentially harbor two different subtypes of HA as extra transcriptional units, opening the possibility for the development of bivalent vaccines that protect poultry against multiple HPAI strains.

One limitation in the use of regular vaccines against AIV or NDV is that vaccinated poultry frequently cannot be differentiated from naturally infected birds, making serological surveillance dif-

ficult to perform. However, vaccination of birds with a bivalent vaccine like rNDV/F3aa-chimericH7, which induces an immune response against H7 HA and NDV in chickens, makes serological surveillance against avian influenza possible because vaccinated animals lack immune responses against antigens present in whole influenza. Therefore, our bivalent vaccine would be suitable for use as a "differentiating infected from vaccinated animals" (DIVA) vaccine. NDV vaccines based on lentogenic strains of NDV are widely used in poultry. A chimeric NDV/AIV bivalent vaccine would be administered in the same way as the conventional NDV vaccines, but would confer protection against both NDV and AIV.

Velogenic NDV strains are able to spread efficiently to neighboring cells upon infection of a variety of cells in the absence of exogenous proteases. This property is partially mediated by the F protein of the virus. The F protein of NDV is synthesized as an inactive precursor (F0) that needs to be cleaved into a heterodimer (F1–F2) to be functional. Cleavage of the F protein is mediated by a host protease that recognizes a cleavage site between the F1 and F2 subunits. When the F1–F2 heterodimer is expressed on the surface of infected cells, it induces fusion between the infected cell and the neighboring cells, resulting in the formation of syncytia. Because the induction of syncytia helps the spread of the virus from cell to cell, the use of a vaccine with a velogenic NDV-derived F cleavage site in poultry may raise potential safety concerns. However, when we introduced a multibasic cleavage site in the F protein of the highly attenuated rNDV/B1, the resulting viruses did not acquire the characteristics typical of velogenic strains with MDTs of $<$60 hr in chicken embryos.

We postulated that enhanced incorporation of the H7 HA protein into rNDV virions may afford a better humoral immune response, resulting in higher levels of protection in chickens against highly pathogenic AIV. However, it was previously observed that virion incorporation of an influenza virus WSN HA protein expressed from rNDV was lower than the homologous HA protein expressed from influenza virus (21). If the H7 HA protein contained the cytoplasmic tail and transmembrane domains of the NDV envelope glycoprotein (F), which are required for efficient viral assembly, in place of its own, this resulted in enhanced incorporation of HA into the viral particle and in improved immunogenicity of the virus. Indeed, the use of an improved NDV vector that had enhanced cell-to-cell transmission and allowed for efficient incorporation of the avian HA into virus particles resulted in 90% protection against challenge with type A HPAI strains and complete protection against velogenic NDV.

In summary, the use of reverse genetics systems for the construction of chimeric influenza or NDV viruses provides a practical strategy for the protection of poultry against existing and newly emerging viruses of HPAI, as well as against NDV. It is expected that these approaches will also prove useful in the development of bivalent vaccines for other pathogens.

Materials and Methods

Cells and Viruses. 293T and Vero cells were maintained in DMEM with 10% FBS. CEF cells, prepared from 10-day-old specific-pathogen-free embryos (Charles River Laboratories, SPAFAS, Preston, CT), and MDCK cells were maintained in Eagle's MEM with 10% FBS. WSN was generated by plasmid-based reverse genetics, as described in ref. 16, and propagated in MDCK cells. We have previously generated a full-length infectious clone of NDV, pT7NDV/B1 (21). rNDV was proliferated in embryonated chicken eggs.

Construction of vRNA Expression Plasmids from Influenza A/Vietnam/1203/04(H5N1). cDNAs derived from influenza A/Vietnam/1203/04(H5N1) RNA (kindly provided by Terrence Tumpey, U.S. Centers for Disease Control and Prevention, Atlanta) by RT-PCR were cloned between a PolI promoter and a hepatitis delta virus ribozyme present in the pPolI-SapI-RT plasmid (28). Full-length cDNAs for seven of the eight viral segments from influenza A/Vietnam/1203/04(H5N1) virus were amplified by PCR with primers that are competent for the subsequent cloning of the full-length viral segments into pPolI-SapI-RT, allowing the production of exact negative-sense vRNAs. In this way, seven segments of influenza A/Vietnam/1203/04(H5N1) virus were cloned into pPolI-SapI-RT. To construct a chimeric vRNA segment encoding the ectodomain of the NDV HN protein (Fig. 1), the region corresponding to the ectodomain of the NDV HN (i.e., amino acids 51–576), followed by tandem stop codons, was amplified by PCR and inserted between the first 127 nucleotides and the last 185 nucleotides of the WSN NA vRNA.

Modification of HA to Remove the Polybasic Cleavage Peptide. pPolIVN1203-HAlo was designed to produce the HA vRNA in which nucleotides 1014–1038 of the HA coding sequence were altered to a consensus sequence that was based on avirulent avian strains of influenza A H5. The nucleotide sequence was altered from CAA AGA GAG AGA AGA AGA AAA AAG AGA GGA to CAG CGG GAG ACG CGG GGA. The codon usage in this region was modified to lessen the presence of adenosine residues in the nucleotide sequence and to minimize opportunity for the reintroduction of adenosine residues by polymerase slippage, and the resultant introduction of basic residues into the HA protein cleavage site (26). Therefore, the encoded amino acid sequence in this region was altered from QRERRRKKR $\downarrow$ G to QRETR $\downarrow$ G (where the arrows indicate cleavage sites).

Generation of Transfectant Influenza Virus. To generate the chimeric influenza virus expressing the NDV HN ectodomain, 1 μg each of 15 plasmids was transfected into 293T cells in monolayer. Each transfection contained vRNA expression plasmids for the A/Vietnam/1203/04 PA, PB1, PB2, HAlo, NP, M, and NS segments; the cHN segment; and the protein expression plasmids pCAGGS-PA, PB1, PB2, NP, HA, NA, and NS1 (pCAGGS expression plasmid kindly provided by J. Miyazaki, Osaka University, Osaka) derived from WSN (29). At 48 hr after transfection, supernatants were harvested, and transfectant virus was passaged into 10-day-old embryonated chicken eggs. RNA segments of transfectant virus were visualized as described in ref. 30.

Generation of rNDVs with a Modified Cleavage Site in Their F Proteins. To generate rNDV/F3aa, two PCR fragments were generated by using primers (regions of overlap are shown underlined) F3aa-1(+), 5′-GGA TCC CGG TTG GCG CCC TCC AGG-3′ with F3aa-1(−), 5′-AAa GCG CCt CTG TCT CCg CCC TCC AGA TGT AGT CAC AG-3′; and F3aa-2(+), 5′-GGc GGA GAC AGa GGC GCt TTA TAG GCG CCA TTA TTG G-3′ with F3aa-2(−) 5′-CCA TAT TCC CAC CAG CTA GAT TGT-3′; and pT7NDV/B1 as the template. The nucleotides shown in lowercase are mutated to modify the amino acid sequence of the cleavage site of the F protein from that of the NDV/B1 strain (GGRQGR $\downarrow$ L) to G*R*RQ*R*R $\downarrow$ *F* (amino acids modified are shown in italics). These overlapping fragments were combined by PCR using primers F3aa-1(+) and F3aa-2(−). The resulting PCR fragment was cloned into pSLF3aa. The StuI–NotI fragment (nucleotides 4646–4952) of pSLF3aa was excised to replace the corresponding fragment in the pT7NDV/B1 plasmid, resulting in the formation of the pT7NDV/F3aa plasmid, which was used to generate rNDV/F3aa virus by reverse genetics. For generation of rNDV/F2aa, PCR mutagenesis was performed by the same strategy described above, with primers F2aa-1(+), 5′-GGA TCC CGG TTG GCG CCC TCC AGG with F2aa-1(−) 5′-AAG GCG CCt CTG TCT CCg CCC TCC AGA TGT AGT CAC AG-3′; and F2aa-2(+), 5′-GGc GGA GAC AGa GGC GCC TTA TAG GCG CCA TTA TTG G-3′ with F2aa-2(−) 5′-CCA TAT TCC CAC CAG CTA GAT TGT-3′; and pT7NDV/B1 as the template. Two overlapping PCR fragments were combined by PCR using primers F2aa-1(+) and F2aa-2(−),

resulting in modification of the cleavage site from GGRQGR ↓ L to G*RRQ*RR ↓ L.

Generation of a Fusogenic rNDV Vector Expressing the Chimeric H7 HA Protein. To construct the chimeric H7 HA gene as an extra transcriptional unit of the rNDV/F3aa, the fragment containing the transmembrane (TM) and the cytoplasmic tail (CYTO) of the NDV F gene was initially produced by PCR using primers HpaNDV F(TM+CYTO)P, 5′-cg GTT AAC CTC ATT ACC TAT ATC GTT TTG ACT-3′; and SacNhe NDVF(TM+CYTO)M, 5′-cg GAG CTC AAG CTA GCT TAT CAC ATT TTT GTA GTG GCT CTC ATC TG-3′; and the NDV F gene as a template. This PCR product was digested with SacI and HpaI and then cloned into the plasmid pNhe-NDV-GE/GS, which possesses the gene end and the gene start signals of NDV, to produce plasmid pNhe-NDV-GE/GS-NDVF(TM+CYTO). As the next step, allowing the connection of the H7 HA ectodomain with the TM and CYTO region of the NDV F, the region encoding the H7 HA ectodomain was amplified by PCR using the primers SpeH7(ECTO)P, 5′-cg ACT AGT CCG CCA CCA TGA ACA CTC AAA TTC TGG CAT TCA T-5′; HpaH7(ECTO)M, 5′-cgG TTA ACG TCT TTG TAT CCA CTA CTC AAT TTC AC-3′; and the H7 HA gene from A/chicken/NY/13142–5/94(H7N2) as the template. This PCR product was digested with SpeI and HpaI and then inserted into the cassette plasmid pNhe-NDV-GE/GS-NDVF(TM+CYTO). In a final step, the cassette plasmid was digested with NheI to cut out the chimeric H7 HA gene. This DNA fragment was cloned between the P and M genes of pT7NDV/F3aa, forming pT7NDV/F3aa-chimericH7. The rNDV/F3aa virus expressing the chimeric H7 HA protein was then rescued from pT7NDV/F3aa-chimericH7 by using reverse genetics (21).

Viral Growth Kinetics. rNDVs (100 plaque-forming units per egg) were inoculated into 10-day-old embryonated chicken eggs. Allantoic fluids were harvested to determine viral titers at different time points (24, 48, and 72 hr). The tissue culture 50% infective dose ($TCID_{50}$) of each virus present in the allantoic fluid was determined by immunofluorescence assay.

Immunofluorescence Assays. MDCK cells infected with transfectant influenza virus were fixed and permeabilized with ice-cold methanol. Viral antigens were detected with anti-NDV HN monoclonal antibody (7B1), anti-influenza H1 HA monoclonal antibody (2G9), and anti-influenza H5 HA polyclonal serum. For the analysis of NDV growth, confluent Vero cells were infected with the rNDVs, which were harvested at 24, 48, and 72 hr. Infected cells were fixed with 2.5% formaldehyde containing 0.1% Triton X-100. Fixed cells were treated with anti-rabbit NDV polyclonal antibody, washed, and stained with FITC-conjugated anti-rabbit immunoglobulins (DAKO).

MDT. To check the pathogenicity of rNDVs in embryonated chicken eggs, MDT was determined. Briefly, five 10-day-old embryonated chicken eggs were infected with serial 10-fold dilutions of viruses. The eggs were incubated at 37°C and monitored two times daily for 7 days. The time to kill embryos was recorded. The highest dilution that killed all embryos was determined to be the minimum lethal dose. The MDT was calculated as the mean time for the minimum lethal dose to kill the embryos.

Western Blotting. Dishes (100 mm in diameter) of confluent Vero cells were infected with rNDVs at a multiplicity of infection of 1. Cell extracts were prepared in RIPA buffer (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) at 36 hr after infection. Equal amounts of protein were analyzed by SDS/PAGE. H7 HA and NDV-derived protein were immunoblotted by using anti-rabbit NDV polyclonal or anti-chicken AIV H7 polyclonal sera. The incorporation of H7 HA protein into virions was also determined by Western blotting. For this Western blot, virus particles from the allantoic fluid of infected embryonated chicken eggs were purified through a sucrose cushion, and the total amount of protein from each virus was measured with the Bradford assay kit (Bio-Rad). Equal amounts of protein were analyzed by immunoblotting as described above.

Immunization and Challenge of Chickens. White Leghorn chickens were vaccinated once or twice by eyedrop in the conjunctival sac with $10^{5.7–6.1}$ mean chicken EID_{50} of rNDV/F3aa-chimericH7, or twice with $10^{5.7–6.3}$ EID_{50} of pNDV, or twice with sterile tissue culture media (sham) at 2 and 4 weeks of age. At 6 weeks of age, the chickens were challenged intranasally with the Fontana strain of vNDV ($10^{5.1}$ EID_{50} per bird) or A/Human/Steele/59 (H7N7) HPAI ($10^{5.1}$ EID_{50} per bird). The survivors were bled and killed at 14 days after challenge. HI serological titers were determined by using standard procedures. All challenge experiments were done at the U.S. Department of Agriculture (USDA) poultry research laboratory in Athens, GA, in accordance with USDA guidelines.

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